CHARLES RIVER LABORATORIES

Transgenic Animal Science: Principles and Methods

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Transgenic Animals and Genetic Research

Prior to the current revolution in applied molecular genetics, the only practical method to study the regulation and function of mammalian genes was to utilize spontaneous mutants. To prove the genetic basis of the mutation, the animals had to transmit an observable trait to offspring, and successful attempts were made to isolate genetic defects on a different genomic background by performing congenic breeding experiments. The major problem with this method of genetic evaluation is that a large amount o f DNA flanking the mutant genetic locus is invariably transferred from animal to animal during meiotic recombination, along with the gene(s) in question.

Since the 1970s, it has been possible to introduce DNA fragments into prokaryotic and eukaryotic cells in vitro and to induce the expression of the foreign DNA in these cells. DNA may be introduced into cells using shock and precipitation, electrical poration of the membranes, viral carriers (i.e., *vectors*) and direct microinjection. Approximately one out of several thousand treated cells usually takes up and *expresses* the foreign DNA; the DNA may be expressed as extrachromosomal satellite DNA or it may be *integrated* into the cellular chromosomes.

Characterized gene sequences may be introduced into cultured cells, and the protein product of the transferred gene, if secreted, may be collected from the culture medium. Although the evaluation of gene expression is relatively straightforward, the activity of a specific gene at the cellular level does not yield satisfactory information about the regulation of the gene among the complex physiological interactions of the whole animal. Current state-of-the-art cell cultures cannot possibly simulate tissu es and organ systems and predict responses to sophisticated environmental stimuli.

The Value of Transgenic Animals

Transgenic animal systems combine the virtues of cell culture and congenic breeding strategies while avoiding the negative aspects of each system. Using transgenic techniques, a characterized genetic sequence may be evaluated within the specific genomic background of the whole animal. Therefore, transgenic animals may be utilized to study the regulation of a specific genetic sequence in a realistic fashion. Many uses have been developed and many more are forecast, particularly in three areas:

Models of human disease processes . Hundreds of transgenic rodent lines have been produced by introducing into the genome genetic sequences such as viral transactivating genes and activated oncogenes implicated in specific pathologies. The phenotype and regulatory parameters of the gene then may be evaluated in an animal model with a relatively short generation time. Also, normal rodent genetics and physiology are highly characterized. The predictability of many transgenic phenotypes permits the i nnovative testing of diagnostic and therapeutic agents while using a reduced population of experimental animals. The generation of novel cell lines from transgenic organs also promises to reduce the number of research animals required to evaluate a therapeutic compound. In addition, transgenic genomes may be created in which more than one transgene may interact, or in which a transgene may interact with an endogenous normal or mutated gene. The use of transgenic disease models in biomedical research promises to accelerate dramatically the development of new human diagnostic and therapeutic treatments. Transgenic rodent models have been characterized for several human diseases including cardio-vascular disease (Walsh et al., 1990), cancer (Sinn et al., 1987), autoimmune disease (Hammer et al., 1990), AIDS (Vogel et al., 1988), sickle cell anemia (Ryan et al., 1990) and neurological disease (Small et al., 1986).

Targeted production of pharmaceutical proteins . Another use for transgenic animals involves the biological production of valuable human protein enzymes, hormones and growth factors. These products may be recombinant or mutated, and collection of the functional protein from the animal employs tissue-specific regulatory DNA sequences, a strategy described below. Current techniques in the biotechnology industry use large-scale cell cultures to generate products in biological systems. Eukaryotic cell s or bacteria which have taken up genetic expression sequences (or *constructs*) are cultured in nutrient medium which is continually replaced and from which the bioengineered product is refined. This medium must be correctly buffered and must be temperature-regulated and maintained pathogen-free. The use of transgenic animals, particularly larger mammals, as bioreactors ("pharmaceutical pharming") is a cost-effective alternative to cell culture methods. Animals automatically supplement thei r bodily fluids with fresh nutrients, remove waste products, reliably regulate their internal temperature and pH and resist pathogens. By directing (or *targeting*) the expression of the transgene product so that it is produced by the secretory cells of the liver, lactating mammary gland or kidney, "pharmers" may collect and process bodily fluids with minimal effort. The mammary gland probably is the most promising target tissue because it produces large amounts of protein in a temperature-r egulated fluid that may be collected daily in a non-invasive fashion. Transgenic animals are not only cost-effective bioreactors but, with the complex secretory cell types and organs of the mammalian organism, can perform much more complicated protein modifications than simply cultured cells.

Modification of animal anatomy and physiology . The most controversial aspect of transgenic animal usage involves the "selective improvement" of species by the modification of the genome. Most often, foreign genes are added to the host genome, but selective deletion of specific genes or regions has been attempted. It has become apparent that merely adding genes for growth factors or hormones to the genome is a simplistic approach to altering the complex multigenic physiology of the mamma l. The goals of this type of experiment may include decreased body fat, increased speed, novel disease resistance or higher yields of meat or milk. At present, these types of phenotypic alterations are more realistically achieved in plants and bacteria than in animals.

Development of Transgenic Science

In the 1970s, experiments were conducted with *embryonal carcinoma* cells and *teratocarcinoma* cells to construct *chimeric* mice (Brinster, 1974; Mintz and Illmensee, 1975; Bradley et al., 1984). In these chimeric animals, cultured cells derived from one strain of mouse were introduced into the embryos of another strain of mouse by direct embryo aggregation or by injection into the blastocyst stage embryo. The adult chimeric mouse could be produced by cellular contributions from several "parents" and would exhibit characteristics of each strain. Another type of animal genome transfer involved the transfer of the entire nucleus from an embryo directly into the enucleated oocyte of a different recipient strain (McGrath and Solter, 1983). These transgenic animals were produced without any recombinant DNA techniques and represent important milestones in the elucidation of genetic regulatory mechanisms in mammalian systems.

The next step in the evolution of transgenic technology was accomplished by the infection of preimplantation mouse embryos with retroviruses (Jaenisch and Mintz, 1974; Jaenisch, 1976). The viral information was successfully transferred into the genome of the recipient animal, and the technique of utilizing retroviruses as vectors for specific foreign DNA sequences was soon developed (Stuhlmann et al., 1984). Retrovirus-mediated transgenesis produces a high degree of mosaicism (discussed below); the size of the transgene sequence is limited, and the viral sequences may interfere with expression of the transgene. However, the integration of single copies of the transgene flanked by the viral DNA can be advantageous if it is desired to clone the locus of integration.

In recent years, several other techniques have been reported to produce transgenic animals. These include embryonic stem cell-mediated techniques (Gossler et al., 1986), transfer of entire chromosomal segments (i.e., "transomic" mice; Richa and Lo, 1988) and gamete transfection in conjunction with in vitro fertilization (Lavitrano et al., 1989). Currently however, the technique of *pronuclear microinjection* is the most successful and most widely-used method of producing transgenic animals. Using this technique, trangene sequences up to about 50 kilobases (kb) in length from viral, prokaryotic, plant, invertebrate or vertebrate sources may be introduced into the mammalian genome, where they may be expressed in both somatic cells and germ cells.

Pronuclear Microinjection

The first successful production of transgenic mice using pronuclear microinjection was reported in 1980 (Gordon et al., 1980). Although the recombinant viral construct was proven to have integrated into the mouse genome, it was rearranged and did not express. Subsequent reports (Brinster et al., 1981; Costantini and Lacy, 1981) proved that integrated transgenes were capable of functional expression following pronuclear microinjection. The first visible phenotypic change in transgenic mice was described in 1982 for animals expressing the rat growth hormone sequence (Palmiter et al., 1982). Currently, several hundred transgenic expression papers are published each year, the majority examining the effects of microinjected viral sequences on mammalian growth and pathology.

The pronuclear microinjection method of producing a transgenic animal results in the introduction of a purified double-stranded DNA sequence into the chromosomes of the fertilized mammalian egg. If this *trans* ferred *gene* tic material (i.e., *transgene*) is integrated into one of the embryonic chromosomes, the animal will be born with a copy of this new information in every cell. The foreign DNA must integrate into the host genome prior to the doubling of genetic material that precedes the first cleavage or a *mosaic* animal may be produced in which many cells do not possess the new gene. For this reason, the transgene DNA is introduced into the zygote at the earliest possible stage, i.e., the *pronuclear* period immediately following fertilization. For several hours following the entry of the sperm into the oocyte, the male and female pronuclei are microscopically visible as individual structures. The transgene may be microinje cted into either of these pronuclei with equivalent results. However, X-chromosome or Y-chromosome integration events do occur and obviously may be influenced by the choice of pronucleus. Usually, the male pronucleus may be distinguished because it is larger than the female nucleus and also because it is closer to the oocyte surface.

The animal that develops after receiving the transgene DNA is referred to as the *founder* (Fo) of a new transgenic lineage. If the germ cells of the founder (mosaic or not) transmit the transgene stably, then all descendants of this animal are members of a unique transgenic lineage. Integration of foreign DNA into the embryonic genome generally is a random event with respect to the chromosomal locus. Therefore the probability of identical integration events in two embryos receiving the same transgene is overwhelmingly unlikely. In addition, it is impossible to regulate exactly how many copies of the transgene will be introduced into the embryo and how many will join together to integrate (usually at a single site) as a single linear array called a *concatamer* (Brinster et al., 1981, 1985; Bishop and Smith, 1989). Many studies have found dramatic differences in the expression of a specific transgene within individual sibling embryos simply due to different integration loci. The num ber of copies of the transgene that have joined the founder's genome is referred to as the *copy number,* and rarely appears to be correlated with the degree of transgene expression in the animal.

Because the locus of transgene integration is random, the transgene frequently inserts into functional genetic sequences. Interruption of the normal expression of an endogenous gene may be inconsequential or lethal. Alternatively, observable *insertional mutagenesis* might be apparent when the insertion interferes with the expression of an endogenous developmentally active gene. These mutations are distinguished from the true transgenic phenotype because only a single lineage exhibits the defect. The mutations can involve any system including the special senses, cardiovascular, neurological and reproductive systems, and severe morphogenetic abnormalities may be observed (Woychik et al., 1985). The identification of the locus of transgene insertion is of great value because it maps the locus of an important endogenous gene.

Because the new transgenic locus is present in only one member of a particular paired chromosome, the genotype of the founder is described as *hemizygous* for the transgene rather than heterozygous. A homozygous genotype, in which a pair of transgene alleles is

present, may be produced by the mating of a pair of hemizygous F1 siblings. Obviously, mating a pair of animals with *identical* transgenes but from *different* founder lineages cannot result in a true homozygote in which independe nt segregation of the loci is predictable.

The success of the microinjection technique relies upon the careful collection of a relatively large group of accurately timed embryos from a reproductively synchronized group of female embryo donors. In addition, the techniques of microinjection and embryo transfer to a suitable recipient female must be mastered. Of course, the combined success of all of these manipulative skills ultimately depends upon the fastidious construction and preparation of the transgene DNA fragments to be injected.

Embryo Collection

The choice of the donor parental strains for production of the pronuclear embryos is a point of extreme proprietary concern to most laboratories. Many factors are cited including the response to superovulation, frequencies of embryo survival following microinjection, size of pronuclei and the incidence of specific pathologies inherent in various strains. The relative merit of inbred versus outbred backgrounds may be important for the evaluation of a specific transgene expression. Other factors may invol ve coat color, the availability of a certain strain, or simply anecdotal rationales. Certain hybrids (e.g., C57BL/6 x SJL/N) and outbreds (e.g., CD-1) are reported to yield large numbers of viable pronuclear embryos following superovulation. The FVB/N inbred strain is reputed to survive microinjection procedures better than many other strains and has been shown to possess pronuclei of relatively larger volumes.

Whichever strain is chosen to provide embryos, fewer animals will be needed and less variability encountered if exogenous gonadotropins are used to *superovulate* the donor females. Successful superovulation protocols must consider the strain, age and weight of the animals. Breeding should be monogamous, and the light cycle in the breeding room must be strictly regulated. The superovulation and synchronization of rats, rabbits and larger mammals present additional technical challenges.

On the morning following breeding, oviducts are removed from euthanized donors, and clumps of pronuclear embryos are collected from the oviducts by flushing or by dissection into a microdrop of sterilized buffered medium. The embryos are clumped together with sticky follicular cumulus cells that must be removed by brief treatment in a series of microdrops. The first drop, a solution of the enzyme hyaluronidase, is followed by two or more wash drops. Using heat-pulled tapered micropipets controlled by mo uth suction (a new pipet for each drop), the embryos are transferred from drop to drop until they are free of cumulus cells, debris and enzyme. Finally, the embryos are transferred into a pool of medium in a petri dish that will be placed under the microscope. The embryo-containing pool is covered by a layer of sterile-filtered, autoclaved mineral oil to prevent contamination by microorganisms and debris and to prohibit evaporation and the resultant pH changes that would kill the embryos. All collection and manipulation media contain a buffering system (i.e., bicarbonate or HEPES) and protein source (e.g., bovine serum albumin) to prevent embryos from adhering to the dishes and pipets. In addition, media may contain antibiotics (e.g., penicillin and/or streptomycin) and a heavy-metal chelating agent (e.g., EDTA).

Equipment

The equipment required to perform microinjection can cost between $50,000 and $80,000 and includes:

- CO_2 incubator to maintain manipulated embryos at 37-38° C in an atmosphere of 5-6 percent CO_2.
- Inverted microscope with a fixed stage.
- Phase contrast, Nomarski differential interference, or Hoffman modulated contrast optical systems to visualize pronuclei. With 10x or 15x eyepieces, a 20x or 40x objective is required.
- A pair of micromanipulators to control the DNA injection pipet and the embryo-holding pipet.
- A pair of micro-volume syringes and associated tubing to regulate the fluid dynamics in the injection and holding pipets. (Expensive automatic microinjection systems are available in lieu of the injection syringe.)
- Pipet-pulling apparatus.
- Vibration-free pneumatic table (optional).
- Microforge apparatus to heat-polish and bend pipets.
- Pipet beveling apparatus (optional).
- Supply of clean capillary pipets for the manufacture of holding and injection pipets.
- Fluorinert solution (optional) to provide optimal fluid dynamics in the pipets.
- Microphotographic equipment (optional) including 35mm camera and/or video recording apparatus.

The foregoing list includes only the supplies required for DNA microinjection. It does not include the animal maintenance supplies (e.g., cages, food, hormones, etc.), or the vast investment in equipment, manpower and supplies necessary for the cloning and preparation of the transgene DNA fragments.

Transgene DNA Preparation

The transgene DNA is engineered in the molecular laboratory to achieve fairly predictable expression in the animal. Using *restriction enzymes* and *ligase,* different functional regions of genes from different species may be recombined in the test tube. All components of endogenous genes may be isolated and recombined to form a *transgene expression cassette* or *construct* (Figure 1). The ends of the completed construct may be modified by the addition of *polylinker* sequences containing several different restriction enzyme recognition sites. The polylinker permits the construct to be inserted into a variety of vectors for testing and cloning. The following review of endogenous gene components will clarify these strategies:

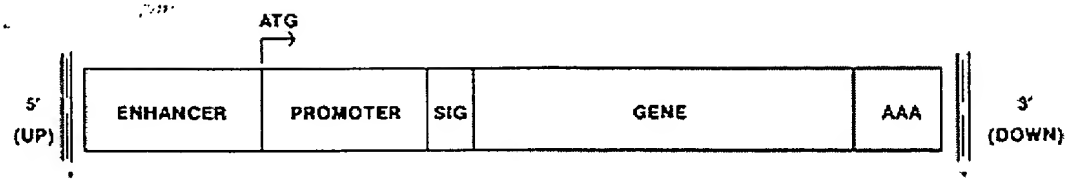

Figure 1 . Schematic diagram of the double-stranded DNA regions of the transgene expression cassette.

* Restriction enzyme recognition sites are clustered at either end of the cassette (i.e., upstream and downstream).
"ATG" indicates the beginning of the transcriptional reading frame.
"SIG" indicates the signal sequence.
"AAA" indicates the poly-A tail

The endogenous gene contains *exons* that code for specific portions of the final protein and *introns* that appear necessary for optimal expression of the gene (Figure 2). The endogenous gene is flanked by non-coding DNA sequences that regulate gene expression. (Regulatory elements may also reside within intragenic intron sequences.) Sequences located at the 5' end of the gene are known as *upstream* elements, while *downstream* elements are found past the 3' end of the gene sequenc e. Regulatory elements called *promoters* are usually found immediately upstream of the gene, and have critical roles in the temporal and tissue-specific regulation of gene expression. Other regulatory elements called *enhancers* function to enhance gene expression, independent of their location and orientation with respect to the gene. Enhancer regions appear to correlate with *DNase hypersensitive sites* and may be several kilobases (kb) distant from the gene. *Signal sequences* are shor t sequences that target protein synthesis into specific intracellular pathways and frequently direct secretion of the protein from the cell. Secretory signals usually are found directly adjacent to the 5' end of the gene, and organelle targeting sequences usually are found within the 3' end of the gene or immediately downstream of the 3' end. These signal sequences are within the *reading frame* or transcriptional region of the gene and therefore encode mRNA and short polypeptide products. The 3' end of the reading frame also must contain a poly-A nucleotide sequence to ensure proper mRNA transcription and translation.

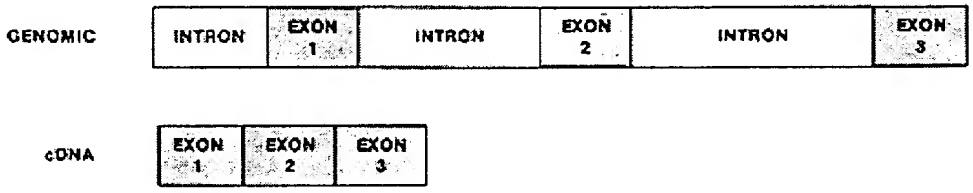

Figure 2 . Comparison of the two forms of transgenes that may be introduced into embryonic pronuclei.

The *genomic* form includes all naturally-occurring *intron* elements that are involved in mRNA splicing and expression, whereas the shorter *cDNA* form is a synthetic sequence representing only the protein-encoding *exon* elements of the gene.

Any transgene fragments used in pronuclear microinjection represent several months or years of intensive efforts by a team of molecular biologists. The transgene cassette is inserted into a *plasmid vector* and *cloned* (copied) within *E. coli* (Figure 3 A-B). The transformed bacteria containing the plasmids are identified by survival selection culture techniques using the antibiotics to which the plasmids are resistant. Surviving bacteria are grown in nutrient medium overnight, and the transgene/plasmid is copied each time the bacteria divide. Several million copies of the transgene-bearing plasmid are then extracted from the bacteria, and the transgene fragments are excised from the plasmids using restriction enzymes. The transgene fragments may be purified by electroelution from electrophoretic gels or by collecting them from a sucrose gradient. The transgene fragments are dissolved in a specific microinjection buffer (i.e., a Tris-EDTA solution), and the DNA concentration is calculate d. The working concentration of DNA for microinjection is usually between 1 and 5 µg/ml. Before introduction into mouse embryos, the transgene construct is tested for expression in cultured cells, usually following a DNA introduction technique known as *transfection* (Figure 3C).

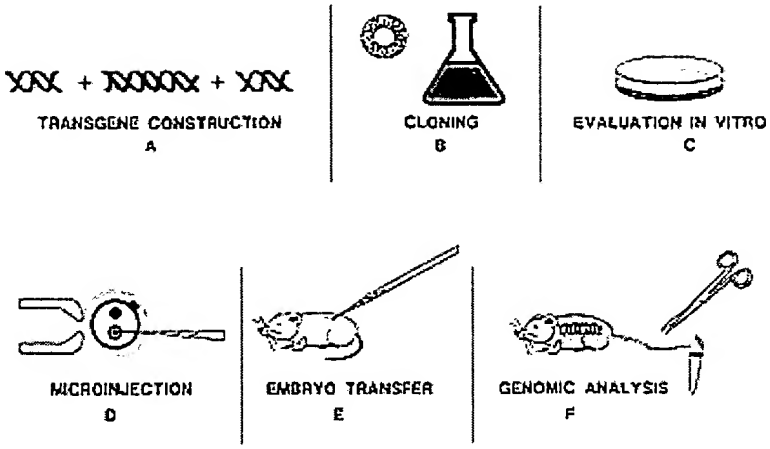

Figure 3. Sequence of events in the generation of a transgenic animal by pronuclear microinjection.

A. The double-stranded DNA components of the transgene are combined enzymatically to yield a transgene expression cassette.
B. Transgene cassettes are inserted into plasmid vectors and cloned.
C. Transgene-bearing plasmids are transfected into cultured eukaryotic cells to evaluate expression of the transgene.
D. Plasmid-free transgene fragments are introduced directly into embryonic pronuclei.
E. Manipulated embryos are placed in the reproductive tract of a pseudopregnant recipient.
F. The genomic DNA of live-born pups is analyzed for the presence of the transgene DNA sequence.

The presence of vector DNA sequences may inhibit expression of the integrated transgene, so this plasmid DNA usually is snipped off with restriction enzymes before purification of the fragments. Also, linear fragments have been observed to integrate at a higher frequency than circular or supercoiled molecules (Brinster et al., 1985). It is apparent from several reports (Brinster et al., 1988; Palmiter et al., 1991) that *genomic* forms of transgenes containing introns or mRNA splice sites can be expected to exhibit greater expression levels than their *cDNA* versions (Figure 2), which merely contain the exon sequences of the gene. The introns may be involved in critical splicing activities and/or regulatory functions.

Microinjection Technique

The pipets used for micromanipulation must be custom-made from thin-walled glass capillary tubing using a pipet puller and a microforge. The holding pipet should have a 15-25 μm internal diameter (embryo diameter = 85 μm). It must be perfectly flat at the tip or much difficulty will be encountered when the embryo is held in place during injection. The DNA injection pipet should have an internal diameter of 1 μm or less and must be tapered toward the end. Each pipet is held in place by an instrument holder and controlled by a separate micromanipulator that regulates movements in three dimensions. Talc-coated gloves should be avoided during all procedures because the powder will clog pipets and may be lethal to embryos.

The petri dish containing the embryo microdrop is placed into focus at a relatively low magnification, and degenerated embryos may be culled from the healthy embryos at this time. The holding pipet is brought down into the medium, and the first embryo is gently sucked onto the end of the pipet and held in place. The tip of the injection pipet is brought into the same plane of focus as the pronucleus to be injected, and a small amount of DNA solution is ejected to ensure the patency of the pipet. The injection pipet is then thrust through the zona pellucida, cell membrane, cytoplasm and nuclear membrane in a single smooth motion (Figure 3D). It is difficult to ascertain visually that the pipet tip has penetrated the pronuclear membrane. Even if the membrane appears to have been pierced, the only reliable indication of success is the swelling of the pronucleus (volume = approximately 1 pl). The pipet is removed smoothly, and the injected embryo is moved to the far end of the pool of medium before the ne xt is processed. Once a group of embryos has been completed, it is transferred in a single volume of medium to another dish for incubation and visual evaluation within a few hours. All apparently viable embryos are then transferred to a recipient female oviduct.

Embryo Transfer

The manipulated embryos must be transferred into a suitable reproductive tract in order to have an opportunity to become live-born transgenic mice (Figure 3E). The recipient female optimally should be somewhat earlier in her reproductive cycle than the embryo donor because manipulated and cultured embryos exhibit slightly retarded development when compared to embryos that developed in vivo. Recipients for embryo transfer are prepared by mating with vasectomized males at the same time that the superovulated donor females are mated with fertile males. It is advisable to use vasectomized males and recipient females with a coat color dominant to the embryo donor so that resources are not wasted testing embryos generated by insidiously fertile vasectomized males. Recipient females are anesthetized, the skin and peritoneum are incised, and the ovarian fat pad and bursa are exteriorized and draped over the midline. The bursa is opened, avoiding any prominent vessels, and the infundibulum is locate d. An embryo transfer pipet with an internal diameter of less than 150 μm is loaded in the following sequence: one small air bubble, approximately 10 μl of medium, a second air bubble, 2-15 embryos in less than 25 μm of medium, and a third air bubble. The pipet tip is inserted into the infundibulum of the oviduct, and the contents are gently transferred

into the oviduct by mouth pressure until the middle air bubble is expelled. The reproductive tract is gently replaced and the incision is closed. Pregnancy shoul d be visible about two weeks after the embryo transfer (post-ET), and the litter should be delivered about three weeks post-ET. Animals may be analyzed for the presence of the transgene in their genomes after weaning at six weeks post-ET (Figure 3F). It should be noted that certain transgene sequences may be activated in utero and may affect embryo survival or gestation length. Also, transgenic females in subsequent generations should be observed for abnormal gestation lengths.

The molecular aspects of transgenics will be reviewed in a future *Technical Bulletin,* which will discuss transgene expression cassettes and regulatory elements, and describe molecular methods used to analyze transgenic animals.

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